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Carbohydrate structure and differential binding of prostate specific antigen to *Maackia amurensis* lectin between prostate cancer and benign prostate hypertrophy

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Serum prostate-specific antigen (PSA) assay is widely used for detection of prostate cancer. Because PSA is also synthesized from normal prostate, false positive diagnosis cannot be avoided by the conventional serum PSA test. To apply the cancer-associated carbohydrate alteration to the improvement of PSA assay, we first elucidated the structures of PSA purified from human seminal fluid. The predominant core structure of *N*-glycans of seminal fluid PSA was a complex type biantennary oligosaccharide and was consistent with the structure reported previously. However, we found the sialic acid α2-3 galactose linkage as an additional terminal carbohydrate structure on seminal fluid PSA. We then analyzed the carbohydrate moiety of serum PSA from the patients with prostate cancer and benign prostate hypertrophy using lectin affinity chromatography. Lectin binding was assessed by lectin affinity column chromatography followed by determining the amount of total and free PSA. Concanavalin A, *Lens culinaris*, *Aleuria aurantia*, *Sambucus nigra*, and *Maackia amurensis* lectins were tested for their binding to the carbohydrates on PSA. Among the lectins examined, the *M. amurensis* agglutinin–bound fraction of free serum PSA is increased in prostate cancer patients compared to benign prostate hypertrophy patients. The binding of PSA to *M. amurensis* agglutinin, which recognizes α2,3-linked sialic acid, was also confirmed by surface plasmon resonance analysis. These results suggest that the differential binding of free serum PSA to *M. amurensis* agglutinin lectin between prostate cancer and benign prostate hypertrophy could be a potential measure for diagnosis of prostate cancer.

Key words: α2,3-linked sialic acid/*Maackia amurensis* agglutinin/prostate-specific antigen/prostate cancer

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Introduction

Prostate-specific antigen (PSA) is recognized as the premier tumor marker for prostate cancer, to which a major cause of death in the male is attributed (Stamey *et al.*, 1987). The usefulness of serum PSA test for early detection and screening of prostate cancer has been well documented (Catalona *et al.*,1994). However, there is an ambiguous area called a diagnostic gray zone where the amount of PSA does not provide a clear distinction between males with benign prostate hypertrophy and those with prostate cancer (Christensson *et al.*, 1993). Although several PSA-related variables such as PSA density (Benson *et al.*, 1992), the rate of PSA secretion (Carter *et al.*, 1995), and free PSA/total PSA ratio (Christensson *et al.*, 1993), have been proposed to overcome this problem, there are still considerable overlaps in those characteristics between the benign and malignant lesions (Vashi *et al.*, 1997).

Because PSA is a serine protease, some PSAs exist in the blood stream in the form of complex with serum protease inhibitors such as α_1-antichymotrypsin and α_2-macroglobulin, whereas the other PSA components exist in a free form (Christensson *et al.*, 1993). The total PSA assay detects both free and PSA-α_1-antichymotrypsin complex. In spite of the application of free PSA as an additional indicator, there is a considerable overlap in a serum PSA concentration between the patients with prostate cancer and benign prostate hypertrophy patients (Vashi *et al.*, 1997). PSA is a glycoprotein containing approximately 8% carbohydrates composed of an *N*-glycan (Bélanger *et al.*, 1995). The structure of carbohydrate on PSA is thought to be a biantennary *N*-linked oligosaccharide of the *N*-acetyllactosamine type (Bélanger *et al.*, 1995; Okada *et al.*, 2001; Peracaula *et al.*, 2003; Prakash and Robbins, 2000; Sumi *et al.*, 1999). Furthermore, it was reported that PSA from the prostate cancer tissues and the prostate cancer cell line (LNCaP) contains *N*-glycans with more antennas than PSA from the benign prostate hypertrophy tissues and the seminal fluid (Prakash and Robbins, 2000; Sumi *et al.*, 1999).

Oncogenesis is accompanied by an alteration in the carbohydrate structure on the cell surface (Fukuda, 1996). Carbohydrates are expressed on the tumor cell surface and involved in the malignant potentials represented by invasion and metastasis (Fukuda, 1996; Ohyama *et al.*, 1999), and a number of carbohydrate tumor markers have been used in the clinical situations. Some glycoprotein tumor markers are also expressed in the normal counterparts, but it is well known that the carbohydrate structure from cancer cells is considerably different from that of nonmalignant cells (Fukuda, 1996). Alpha-fetoprotein and

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human chorionic gonadotrophin are typical examples of this event, where the difference of binding for a specific lectin is used for discriminating benign lesions from cancers (Amano *et al.*, 1988; Yamashita *et al.*, 1993). As for PSA, however, few studies have been performed because of the difficulty in obtaining sufficient amounts of it for carbohydrate structural analyses. Specifically, carbohydrate alteration in serum PSA from cancer patients has not been demonstrated. This may be partly due to the molecular diversity of serum PSA, which contains both free PSA and PSA-α_1-antichymotrypsin complex (Christensson *et al.*, 1993).

In the present study, we performed a systematic carbohydrate structural analysis on the human seminal fluid PSA, and demonstrated the existence of sialic acid α2-3-galactose linkage in addition to the sialic acid α2-6 linkages, which had been considered the sole sialic acid linkage in most of the previous studies. Considering that cancer-associated carbohydrate alterations have been detected by lectin affinity chromatography of PSA (Prakash and Robbins, 2000; Sumi *et al.*, 1999), we tested several different lectins to develop an improved assay system for discriminating cancer-associated PSA from that of benign lesions. We found that free serum PSA from prostate cancer patients exhibits increased binding to *Maackia amurensis* agglutinin (MAA) than that from benign prostate hypertrophy patients, suggesting that the MAA binding of serum PSA could be used as a novel diagnostic measure for prostate cancer.

Results

Structural analysis of fluorescently labeled sugar chains from PSA

The *N*-glycans from the human seminal fluid PSA were cleaved by *N*-glycanase. The released *N*-glycans were pyridylaminated and separated according to their charges by an anion exchange chromatography into neutral and three acidic fractions. They were designated as N, M, D, and T, eluting at the positions of neutral, mono-, di-, and trisialo oligosaccharides, respectively (Figure 1). The ratio of these fractions calculated based on the peak area was N:M:D:T = 42:33:24:1. The minimal amount of T fraction suggested that only a small amount of triantennary or tetraantennary structure was present. The N, M, and D fractions were pooled after multiple separations and then separated by reverse phase high-performance liquid chromatography (HPLC) as shown in Figure 2. This chromatography allowed identifying the structure of those *N*-glycans by comparing their elution positions with those of standard *N*-glycans.

The deduced structures of the neutral sugar chains, N9, N8, N7, and N6, were complex biantennary structures with a core fucose consisting of 2, 2, 1, and 0 of nonreducing terminal galactose residues, respectively (Table I). The peaks N4, N3, and N1 were the nonfucosylated form of N9, N8 (7), and N6, respectively. These structures were confirmed by exo-glycosidase digestion using β-galactosidase and β-hexosaminidase, and the molecular mass measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), as shown in Table I.

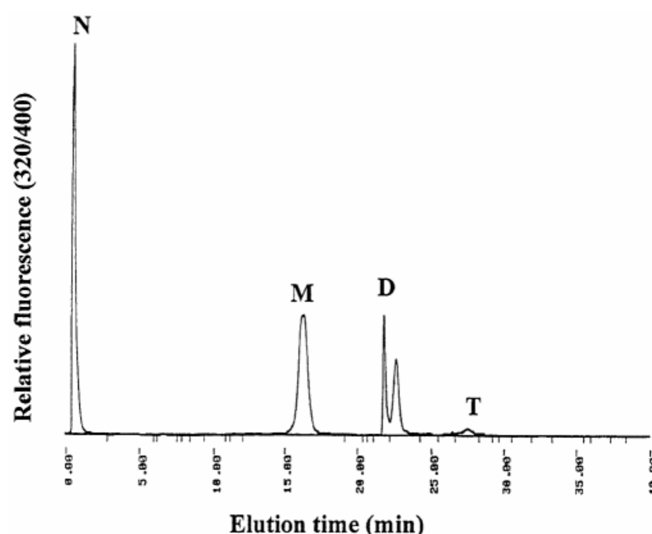

Fig. 1. Anion exchange HPLC of *N*-glycans-PA from human seminal fluid PSA. The *N*-glycans from human seminal fluid PSA were pyridylaminated and fractionated into neutral (N), mono- (M), di- (D), and tri- (T)sialo oligosaccharides using the experimental conditions described in *Materials and methods.*

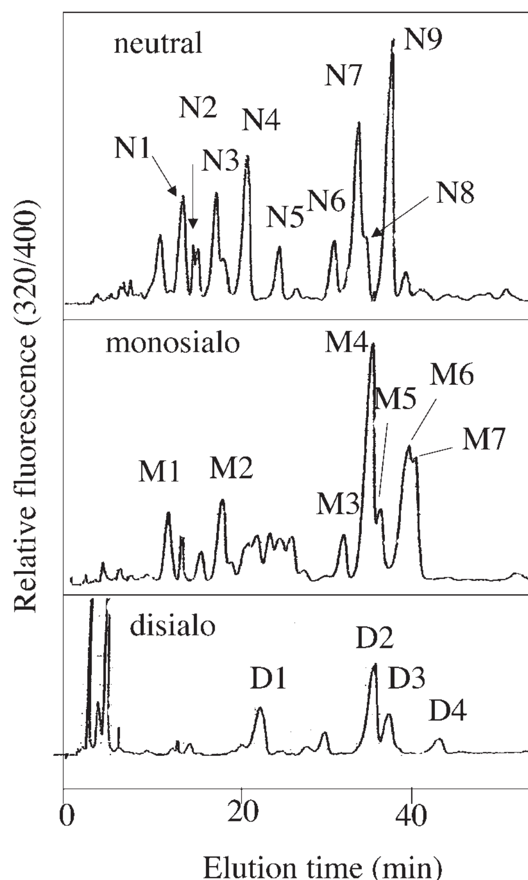

Fig. 2. Reverse phase HPLC of *N*-glycans-PA from human seminal fluid PSA. The neutral, monosialo, and disialo fractions were accumulated by multiple separations using the procedure shown in Figure 1 and then separated by reverse phase HPLC using the elution condition described in *Materials and Methods.*

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Table I. MS data of prostate-specific antigen *N*-glycans separated by HPLC

$[M+Na]^+$	Pyridylaminated *N*-glycans composition	NANase I digest		NANase II digest		
		$[M+Na]^+$	Peak	$[M+Na]^+$	Composition	Peak
1997	$NeuAcHex_6HexNAc_3$-PA	1998	ND[a]	1704	$Hex_6HexNAc_3$-PA	N1
2036	$NeuAcHex_5HexNAc_4$-PA	2035	ND	1745	$Hex_5HexNAc_4$-PA	N4 (N5)
2022	$NeuAcFucHex_4HexNAc_4$-PA	2021	M3	1728	$FucHex_4HexNAc_4$-PA	N8
2183	$NeuAcFucHex_5HexNAc_4$-PA	2203[b]		1890	$FucHex_5HexNAc_4$-PA	N9
2020	$NeuAcFucHex_4HexNAc_4$-PA	2020	M4	1729	$FucHex_4HexNAc_4$-PA	N7
2020	$NeuAcFucHex_4HexNAc_4$-PA	1729	ND	1727	$FucHex_4HexNAc_4$-PA	N7
2184	$NeuAcFucHex_5HexNAc_4$-PA	2181	ND	1889	$FucHex_5HexNAc_4$-PA	N9
2183	$NeuAcFucHex_5HexNAc_4$-PA	1890	ND	1890	$FucHex_5HexNAc_4$-PA	N9
2324	$NeuAc_2Hex_5HexNAc_4$-PA	N.D.	D1	1745	$Hex_5HexNAc_4$-PA	N4
2476	$NeuAc_2FucHex_5HexNAc_4$-PA	2472	D2	1891	$FucHex_5HexNAc_4$-PA	N9
2477	$NeuAc_2FucHex_5HexNAc_4$-PA	ND	N9	1891	$FucHex_5HexNAc_4$-PA	N9
ND		ND	M4	N.D.	$FucHex_5HexNAc_4$-PA	N9

[a]Not determined.
[b][M + 2Na] *m/z*.

N8 has a galactose residue, but N4 does not. A monoantennary structure and hybrid type *N*-glycans, Gal-GlcNAc-$(Man)_3$-$(GlcNAc)_2$-pyridylamine (PA) and Gal-GlcNAc-$(Man)_5$-$(GlcNAc)_2$-PA, were detected in peaks N1 and N2, respectively, of which structures were confirmed by MALDI-TOF analysis and exo-glycosidase digestion (Table I).

Each acidic PA-sugar chain from fractions M and D was separately digested with NANase I and NANase II, and the resultant digests were analyzed by reversed phase HPLC and MALDI-TOF MS (Table I). It has been shown NANase I cleaves α2,3-linked sialic acid, whereas NANase II cleaves both α2,6-linked and α2,3-linked sialic acid (Angata *et al*., 1998). After NANase I digestion, ~40% of *N*-glycans in fractions M and D was converted to *N*-glycans lacking sialic acid ($[M+Na]^+ = 1889$–1991, N9) and a small portion of *N*-glycans in fraction D was converted to monosialyl *N*-glycans (D1). The remaining *N*-glycans were converted to N5, N8, and N9 by NANase II digestion (Tables I and II). In separate experiments, all sialylated *N*-glycans were digested by NANase I or NANase II. The results showed that as an aggregate, the ratio of the sialic acid linkages of α2-6 and α2-3 is approximately 3:1. The predominant core structure of *N*-glycans of the seminal fluid PSA was a complex type biantennary oligosaccharide, which was consistent with the structure reported previously by others (Bélanger *et al*., 1995; Okada *et al*., 2001; Prakash and Robbins, 2000; Sumi *et al*., 1999). The present analysis, however, detected the α2,3-linked sialic acid attached to galactose residue in monosialylated *N*-glycans and mostly in disialylated *N*-glycans, which is consistent with the results obtained previously (Peracaula *et al*., 2003).

Carbohydrate alterations of human serum PSA indicated by lectin column chromatography

For this analysis, the serum samples from these patients were first fractionated by lectin column chromatography and each amount of total and free PSAs in the eluate was measured. Conacanavalin A (Con A) was used for estimating the core carbohydrate structure. MAA specifically recognizes the sialic acid α2-3 galactose residue (Wang and Cummings, 1988), and *Sambucus nigra* agglutinin (SNA) recognizes the sialic acid α2-6 galactose residue (Shibuya *et al*., 1987). *Aleuria aurantia* lectin (AAL) (Kobata and Yamashita, 1993) and *Lens culinaris* (LcH) (Yamashita *et al*., 1993) were used for detecting fucose residues.

Con A column chromatography followed by the PSA assay showed that the relative amount of branched *N*-glycans containing more than two antennas (Con A–unbound fraction) of free PSA was significantly higher in prostate cancer patients than that in benign prostate hypertrophy patients ($p < 0.05$) (Figure 3). In contrast, the high-mannose fraction of free PSA was significantly higher in benign prostate hypertrophy patients than that in prostate cancer patients ($p < 0.05$). There were no significant differences in biantennary and hybrid fractions in free PSA between prostate cancer and benign prostate hypertrophy patients.

It is noteworthy that PSA from benign prostate hypertrophy patients contain approximately 50% of Con A–unbound *N*-glycans. By contrast, PSA from pooled seminal fluid from different individuals contained only small amounts of complex *N*-glycans that were not bound to Con A–agarose column. This suggests that complex N-glycans may be increased even in benign prostate hypertrophy patients. Because only limited amount of *N*-glycans was available, extensive analysis of *N*-glycans using MALDI-TOF MS was not carried out on PSA derived from patients. We thus decided to analyze those *N*-glycans using lectin affinity chromatography.

Among the lectins examined, the MAA-bound fraction of free PSA in prostate cancer patients was significantly higher than that in benign prostate hypertrophy patients

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Table II. Structures and relative amount of *N*-glycans of human PSA from seminal fluid

	Carbohydrate Structures	Neutral	Monosialo		Disialo		
			α2-3	α2-6	α2-3+ α2-3	α2-3+ α2-6	α2-6+ α2-6
		(%)	(%)	(%)	(%)	(%)	(%)
N1[a]	Man╲ Man-GlcNAc-GlcNAc-PA Gal-GlcNAc-Man╱	0.6	-	-	-	-	-
N2	Man╲ Man╲ Man╱ Man-GlcNAc-GlcNAc-PA ±NAN-Gal-GlcNAc-Man╱	3.0	-	2.6	-	-	-
N6	Man╲ Fuc Man-GlcNAc-GlcNAc-PA Gal-GlcNAc-Man╱	2.0	-	-	-	-	-
	GlcNAc-Man╲ Man-GlcNAc-GlcNAc-PA GlcNAc-Man╱	1.8	-	-	-	-	-
N7	GlcNAc-Man╲ Fuc Man-GlcNAc-GlcNAc-PA GlcNAc-Man╱	2.5	-	-	-	-	-
N3	Gal{GlcNAc-Man╲ Man-GlcNAc-GlcNAc-PA GlcNAc-Man╱}	4.5	-	-	-	-	-
N8	±NAN-Gal{GlcNAc-Man╲ Fuc Man-GlcNAc-GlcNAc-PA GlcNAc-Man╱}	9.4	2.9	7.6	-	-	-
	±NAN-Gal-GlcNAc-Man╲ Man-GlcNAc-GlcNAc-PA ±NAN-Gal-GlcNAc-Man╱	6.3	-	3.8	-	-	6.2
N9	±NAN-Gal-GlcNAc-Man╲ Fuc Man-GlcNAc-GlcNAc-PA ±NAN-Gal-GlcNAc-Man╱	11.7	4.6	11.6	5.4	1.8	11.6

[a]Those peak numbers are for neutral *N*-glycans after removal of sialic acid and *N*-glycans containing more than two antennas are not included.

($p < 0.001$) (Figure 4). However the difference in the MAA-bound fraction of total PSA from prostate cancer patients and benign prostate hypertrophy patients was not significant. On the other hand, the SNA-bound fraction of free PSA in benign prostate hypertrophy patients was significantly more than that in prostate cancer patients ($p < 0.05$) (Figure 4). These results suggest that in prostate cancer α2,3-linked sialic acid replaced some α2,6-linked sialic acid in PSA *N*-glycans. There were no significant differences in the bound fraction of AAL and LcH lectins between prostate cancer and benign prostate hypertrophy patients (Figure 4). These results combined indicate that the amount of α2,3-linked sialic acid is increased in free PSA derived from prostate cancer patients compared to benign prostate hypertrophy. Conversely, α2,6-linked sialic acid in free PSA decreased in prostate cancer patients compared to benign prostate hypertrophy.

Surface plasmon resonance analysis

As shown in Figure 5, there was no remarkable difference in the sensorgrams of SNA against PSA-positive proteins from LNCaP culture medium, seminal fluid PSA, and BP-C5 (benign prostate hypertrophy tissue). However, the

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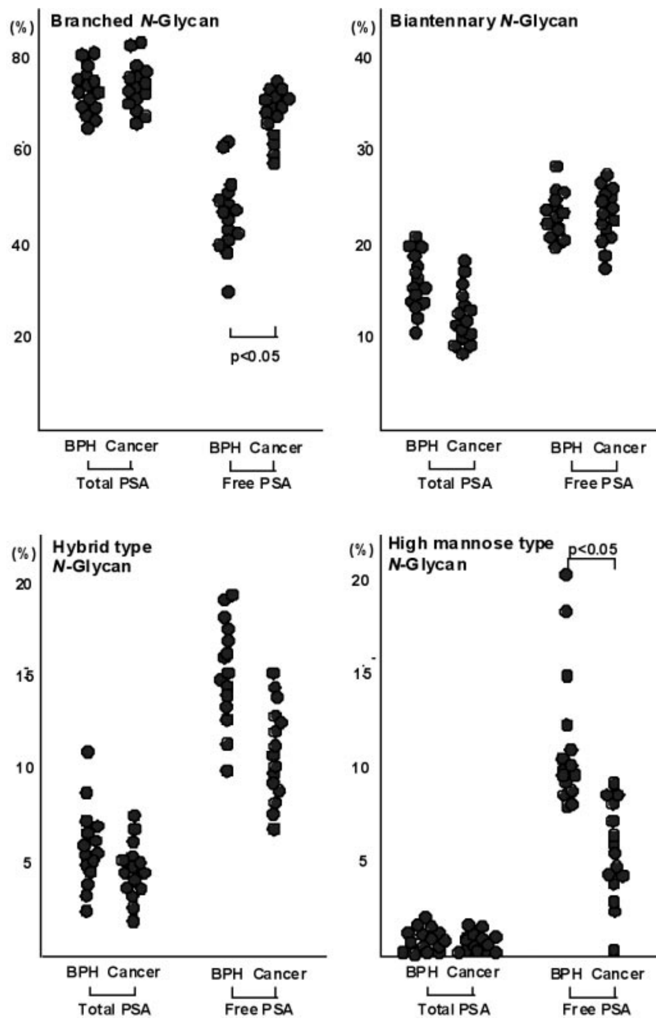

Fig. 3. Rough estimation of core *N*-glycan structures of serum PSA by Con A column chromatography followed by PSA assay. Serum proteins were separated by Con A column chromatography. The amount of free PSA and complexed PSA in the eluates was then determined by enzyme-linked immunosorbent assay. Free PSA from cancer patient is rich in branched *N*-glycans (Con A–unbound fraction). Free PSA from benign prostate hypertrophy (BPH) patient is rich in high-mannose type *N*-glycans.

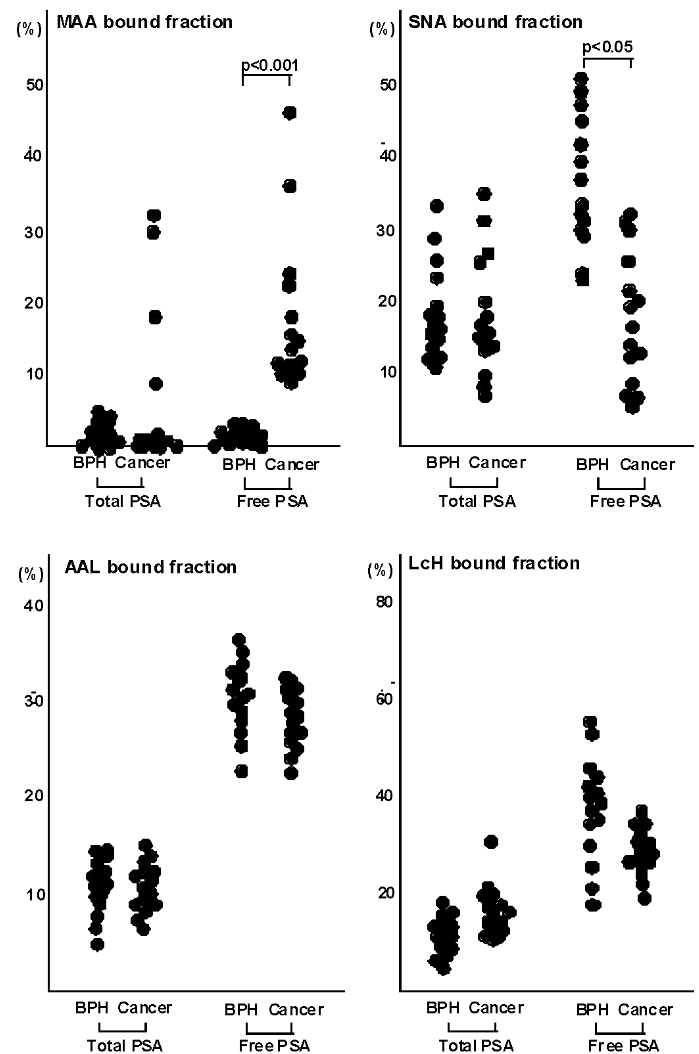

Fig. 4. Relative amount of MAA, SNA, LcH, and AAL lectin–bound fractions of serum PSA from prostate cancer and benign prostate hypertrophy patients. Serum proteins were separated by lectin column chromatography and free and complexed PSA were assayed as described in *Materials and methods*. MAA-bound fraction of free PSA from prostate cancer patients is significantly higher than that from benign prostate hypertrophy (BPH) patients. By contrast, SNA-bound fraction of free PSA from benign prostate hypertrophy patients is significantly higher than that from the prostate cancer patient. There was no significant difference in LcH and AAL lectin bound fractions of PSA from prostate cancer and benign prostate hypertrophy patients.

signal intensity of MAA against PSA protein from LNCaP was more than twice as much as those against the seminal fluid PSA and BP-C5. The surface plasmon resonance analysis indicated that sialic acid α2-6 epitope recognized by SNA was also present in LNCaP culture medium, seminal fluid PSA, and BP-C5, respectively. These results indicate that PSA from prostate cancer LNCaP cells had a larger amount of sialic acid α2-3 galactose linkage than that from the benign prostate tissue and normal seminal fluid.

Although 20% of PSA from seminal fluid and benign prostate hypertrophy patients were found to contain α2, 3-linked sialic acid (Table II and Figure 5), they were not bound to MAA-agarose column (Figure 4). This suggests that a cluster of α2,3-linked sialic acid may be necessary for efficient binding to MAA-agarose column.

Discussion

The serum PSA test is widely accepted as a powerful tool for diagnosis and monitoring patients with prostate cancer. However, there is a considerable overlap in PSA concentrations between prostate cancer patients and benign prostate hypertrophy patients (Christensson *et al.*, 1993; Vashi *et al.*, 1997). The clinical use of the total PSA test has shown that males with PSA levels of 0–4.0 ng/ml and nonsuspicious lesions by digital rectal examination may not need to be examined for further invasive examinations, such as core needle biopsy (Catalona *et al.*, 1994). The patients with a PSA level of between 4.0 and 10.0 ng/ml are classified into

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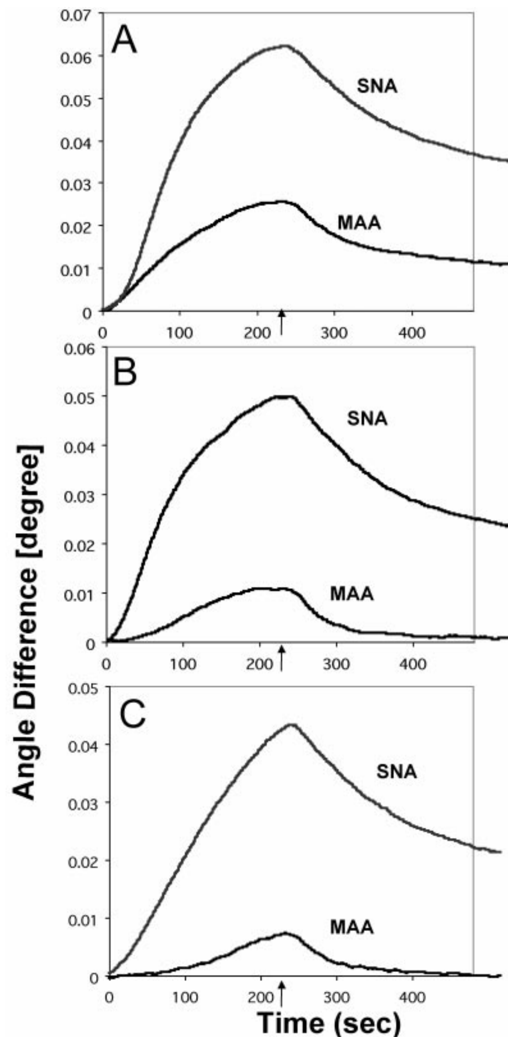

Fig. 5. Binding profiles of SNA and MAA to the PSA from LNCaP culture medium, benign prostate hypertrophy tissue, and seminal fluid. The PSA was immobilized to the sensor chip as described in *Materials and methods*. Overlay plots are shown for SNA and MAA binding to PSA from LNCaP culture medium (**A**), benign prostate hypertrophy (BPH) tissue (**B**), and seminal fluid PSA (**C**). The association (from 0 to 240 s, noted by arrow) and dissociation (after 240 s) profiles of SNA (500 μg/ml) and MAA (500 μg/ml) were analyzed on the same chip using surface plasmon resonance procedure.

the category of the diagnostic gray zone (Christensson *et al.*, 1993). Among those whose PSA levels are in this gray zone, 22.4–26.4% have been diagnosed as having prostate cancer (Catalona *et al.*, 1994). This means that the other 75% of these patients do not have cancer but must endure the discomfort, cost, and anxiety of the prostate biopsy. It has been thus imperative to obtain further biochemical characteristics of PSA to improve the efficiency in discrimination of prostate cancer from benign prostate hypertrophy, relieving patients from the unnecessary invasive examinations.

PSA is a glycoprotein with one asparagine (*N*)-linked oligosaccharide, and it is well known that malignant transformation is often associated with an aberrant glycosylation on the cell surface (Fukuda, 1996). Previously, it has been demonstrated that the cancer-associated alteration of the *N*-glycans on PSA was characterized by an increase in highly branched oligosaccharides (Prakash and Robbins, 2000; Sumi *et al.*, 1999). This is consistent with the findings that the increased branching of *N*-glycans, which is formed by *N*-acetylglucosaminlytransferase V (GnT-V), was associated with cancer cells (Dennis *et al.*, 1987; Hubbard, 1987; Pierce and Arango, 1986; Saitoh *et al.*, 1993; Yamashita *et al.*, 1984). Although the significance of sialic acid residue for the cancer-associated alteration of carbohydrates on PSA has also been suggested (Huber *et al.*, 1995), the precise terminal sialyl structure of PSA carbohydrates still remain to be elucidated. In the present study, we showed that the major core structure of *N*-glycans on the seminal fluid PSA, the normal counterpart of cancer-associated PSA, is biantennary *N*-glycans. This is consistent in principle with the previous reports showing biantennary *N*-glycans in PSA (Bélanger *et al.*, 1995; Okada *et al.*, 2001; Prakash and Robbins, 2000; Sumi *et al.*, 1999). In addition, we revealed the terminal sialic acid linkage as a mixture of sialic acid α2-6 galactose and sialic acid α2-3 galactose with a 3:1 ratio.

Previously, the carbohydrate structure of human seminal PSA was investigated to detect only two components that are biantennary *N*-glycans with or without a fucose (Bélanger *et al.*, 1995). Although the exact reason is unknown, the discrepancy between their results and ours may be due to, at least in part, the difference in the analyzing methods used. Bélanger *et al.* (1995) performed the structural analysis of carbohydrates using nuclear magnetic resonance. Because this technique requires a large amount of samples, the minor components might have escaped from their analysis. In the present study, pyridylaminated *N*-glycans were analyzed by HPLC followed by two kinds of sialidase digestion for determination of the terminal sialic acid linkage. This process facilitated the precise structural analysis because different *N*-glycans can be distinguished using relatively small amounts of materials.

In our study, the statistically significant differences in the core carbohydrate structures of free serum PSA (branched *N*-glycans and high-mannose *N*-glycans) were observed between prostate cancer patients and benign prostate hypertrophy patients (Figure 3). However, there still were considerable overlaps. By contrast, we discovered that MAA-bound fraction of free PSA is significantly increased in prostate cancer patients compared with benign prostate hypertrophy patients, showing a distinct difference with no overlap between the two groups (Figure 4). Because MAA lectin specifically reacts with sialic acid α2-3 galactose terminal structure (Wang and Cummings, 1988), this characteristic carbohydrate moiety of free serum PSA reflects a distinctive increase of sialic acid α2-3 galactose residue in the sera from prostate cancer patients.

It has been reported that PSA purified from LNCap prostate cancer cells contain poorly sialylated *N*-glycans (Peracaula *et al.*, 2003). In contrast, PSA from prostate cancer patients contains increased α2,3-linked sialic acid, as shown in the present study. Although it is not clear why this discrepancy was obtained, it is possible that the LNCap cell line used in their study may be deviated from the parent cell line after many passages.

By contrast, unlike free PSA, total PSA failed to show any significant difference in MAA binding between prostate

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cancer and benign prostate hypertrophy patients. This discrepancy might be attributable to the presence of serum protease inhibitors. α_1-Antichymotrypsin is a glycoprotein that contains bi- and triantennary *N*-glycans (Laine *et al.*, 1991). The lectin-bound fraction assayed by the total PSA detection kit may be affected by the carbohydrates attached to α_1-antichymotrypsin, obscuring the difference in *N*-glycans attached to PSA between prostate cancer and benign prostate hypertrophy patients.

In conclusion, our present study showed that sialic acid α2-3 galactose residue coexists with sialic acid α2-6 galactose residue in the seminal fluid PSA and that there is a distinct increased binding to MAA lectin in free serum PSA isolated from prostate cancer patients compared to benign prostate hypertrophy patients. The distinction can be simply achieved by MAA affinity chromatography followed by estimating an amount of free and complex PSA by enzyme-linked immunosorbent assay. Although further studies are needed to define the clinical application, the MAA-bound fraction of free PSA may provide a promising diagnostic tool for a better discrimination of prostate cancer from benign prostate hypertrophy.

Materials and methods

Human PSA from seminal fluid

The human PSA purified from seminal fluid was purchased from Scripps Laboratory (San Diego, CA).

Prostate cancer cell line

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Rockville, MD) and maintained with RPMI 1640 medium with 10% fetal calf serum. The culture medium after culturing in the serum-free medium (Opti-MEM, Gibco-BRL, Grand Island, NY) for 48 h was used as a source of the cancer-associated PSA for lectin binding assays.

Human benign prostate hypertrophy tissue

The human benign prostate hypertrophy tissue was obtained from a patient who underwent retropubic prostatectomy at the Department of Urology, Sendai National Hospital (Sendai, Japan). Histopathologic examination proved no malignancy in the surgical specimen.

Human serum samples

Serum samples were obtained from 15 randomly selected patients with newly diagnosed prostate cancer at various clinical stages (4 cases at T2N0M0, 5 at T3N0M0, and 6 at T3N1M1) and 15 patients with benign prostate hypertrophy whose total serum PSA levels were between 4.0 and 10.0 ng/ml. Clinical tumor staging was based on the *TNM Atlas* (4th edition, International Union against Cancer, 1997). These patients were treated at the Department of Urology, Sendai National Hospital. The diagnosis for all of the patients was confirmed by the histopathologic findings of the transurethral resection or prostate needle biopsy. The median age of patients was 72 years with a range of 55–82 years for prostate cancer patients and 69 years with a range of 52–81 years for benign prostate hypertrophy patients. The mean total PSA concentration of the prostate cancer patients and the benign prostate hypertrophy patients was 89 ng/ml (range 5.0–128.0 ng/ml) and 8.8 ng/ml (range 5.0–10.0 ng/ml), respectively. Informed consent was obtained from all patients who provided the human materials.

Preparation of PA-N-glycans from human PSA

A complex type biantennary *N*-glycan-PA was prepared from the recombinant human erythropoietin as described previously (Kanazawa *et al.*, 1999). The human seminal fluid PSA (300 μg) was denatured by boiling for 3 min in 240 μl 50 mM Tris–HCl buffer, pH 7.0, containing 0.5% of sodium dodecyl sulfate and 1% 2-mercaptoethanol. After cooling down, 120 μl 7.5% NP-40 was added to the solution, and PSA was digested with 5 U N-glycosidase F (Boehringer Mannheim, Mannheim, Germany) at 37°C for 16 h. The completion of the reaction was confirmed by its migration on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The released *N*-glycans were separated from the protein portion and detergents by using reverse phase HPLC column (Vydac C4, 0.46×250 mm) equilibrated with 12% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The unbound fraction was pooled and then lyophilized. The residue was dissolved in an aliquot of water and applied to a Bio-Gel P-4 column (1.0×45 cm, BioRad, Hercules, CA) equilibrated with 10 mM ammonium acetate buffer, pH 6, and the orcinol-positive fractions were collected and lyophilized. *N*-glycans thus obtained were pyridylaminated by the method described previously (Kuraya and Hase, 1992). Excess reagents were removed by gel filtration using a HW-40F column (1.5×45 cm, BioRad) equilibrated with 10 mM ammonium acetate, pH 6.2.

HPLC

Anion exchange chromatography was performed using a Mono Q column (5 mm $\times$ 50 mm, Pharmacia, Basking Ridge, NJ) equilibrated with 10 mM ammonium in water, pH 9, at a flow rate of 1.0 ml/min at 25°C. After applying PA-glycans from human PSA to the column, PA-glycans bound to the column were eluted by increasing the concentration of ammonium acetate, pH 8.0, from 0 to 0.2 M in 40 min. PA-glycans were detected by fluorescence (excitation wavelength, 310 nm; emission wavelength, 380 nm).

Reverse phase HPLC was carried out using a Shimpack CLC-ODS column (6×150 mm, Shimadzu, Tokyo) equilibrated with 0.05% 1-butanol in 10 mM phosphate buffer, pH 3.8, at a flow rate of 1.0 ml/min at 55°C. After injecting a sample, the concentration of 1-butanol was raised linearly to 0.5% over 60 min. PA-sugar chains were monitored by measuring the fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

PA-glycans fractionated by anion exchange HPLC were digested with two kinds of sialidases; NANase I (Glyko, Novato, CA), which releases only α2-3-linked sialic acid and NANase II (Glyko) which releases both α2-3- and α2-6-linked sialic acids from glycans. PA-sugar chains were digested with 10 mU NANase I or 20 mU NANase II in 50 mM sodium phosphate buffer, pH 6.0, at 37°C for 16 h. Neutral PA-glycans were incubated with 20 mU of jack

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bean β-galactosidase (Seikagaku, Tokyo) in 0.1 M citrate phosphate buffer, pH 4.0, or with 20 mU jack bean β-*N*-acetylhexosaminidase (Seikagaku) in 0.1 M citrate phosphate buffer, pH 5.0, at 37°C for 16 h.

MALDI-TOF MS

MALDI-TOF mass spectra were acquired on a Voyager DE STR (Perceptive Biosystems, Albertville, MN) equipped with a nitrogen laser. Spectra were obtained in the linear mode at an accelerating voltage of 20 kV. The delayed extraction was used with a nominal pulse delay of 200 ns. As the matrix, 2,5-dihydroxybenzoic acid in water (15 mg/ml) was used for all samples. Equal volumes (5 μl) of the sample and matrix solution were mixed, from which 1 μl was applied on a sample plate. Each spectrum obtained was the average of 150–200 scans. The oligosaccharides produced sodium adduct ions, and $[M+Na]^+$ or $[M+2Na]^{2+}$ were detected in a positive ion mode.

Lectin column chromatography and PSA assay

Con A, LcH, AAL, SNA, and MAA conjugated to agarose were purchased from EY Laboratories (San Mateo, CA). Lectin affinity column chromatography was performed as described previously with some modifications (Barak *et al.*, 1989; Chan and Gao, 1991; Kobata and Yamashita, 1993). Briefly, columns of agarose-bound lectins were equilibrated with buffers containing 0.02% Tween 20, 0.02% sodium azide, and 0.5% bovine serum albumin. Serum samples were applied to the column and kept at room temperature for 30 min, followed by washing with five column volumes of the buffer. This fraction was designated as the lectin-unbound fraction. The lectin-bound fraction was eluted with five column volumes of hapten sugars (see following description of methods). For the precolumn, unbound, and bound fractions, total PSA and free PSA were measured by DPC Immulite HS-PSA and DPC Immulite free-PSA (Diagnostic Products, Los Angeles, CA), respectively. Total PSA includes PSA associated with serum protease inhibitors, whereas free PSA does not (Christensson *et al.*, 1993). The relative amount of the lectin-bound fraction against the precolumn amount was calculated.

For Con A column chromatography, samples were separated into four fractions: the unbound fraction (first fraction), the fraction eluted with 10 mM α-methyl glucose (second fraction), the fraction eluted with 10 mM α-methyl mannose (third fraction), and the fraction eluted with 0.3 M α-methyl mannose (fourth fraction). Because PSA has no *O*-glycosylation site but one *N*-glycosylation site (Bélanger *et al.*, 1995), branched *N*-glycans containing more than two antennae are eluted in the first fraction, biantennary *N*-glycans in the second fraction, hybrid type *N*-glycans in the third fraction, and high-mannose type *N*-glycans in the fourth fraction, respectively. The percentage of each fraction relative to the precolumn amount was calculated. Statistical analysis was carried out by Mann-Whitney's *U*-test.

Preparation of PSA-positive fractions from benign prostate hypertrophy tissue and LNCaP culture medium

The prostate tissue from human benign prostate hypertrophy was minced and homogenized with 20 mM phosphate buffer, pH 7.4. Homogenate was centrifuged at $22{,}000 \times g$ for 20 min, and the supernatant was collected. The tissue extract or LNCaP culture medium was fractionated by sequential precipitation with 25% and 70% saturated ammonium sulfate, and the resulting precipitate was dialyzed against 20 mM acetate buffer, pH 5.6. Each of the crude fractions was applied to a CM-Toyopearl cation exchange column (Tosoh, Tokyo) equilibrated with 20 mM acetate buffer, pH 5.6. After the pass-through fraction was washed with the starting buffer (300 ml), the adsorbed proteins were eluted with a linear gradient from 0 to 0.5 M of NaCl. Fractions containing proteins detected by absorbance at 280 nm were combined and subjected to the analysis for detecting the presence of PSA by immunoblotting method. After each sample (total of 3–5 mg protein) was separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% separation gel), the gel was blotted to polyvinylidene difluoride membrane (Immobilon-P^{SQ}, Millipore, Bedford, MA). The membrane was incubated with rabbit anti-human PSA antibody (Dako, Copenhagen), followed by horseradish peroxidase–labeled anti-rabbit IgG antibody (Cedarlane Lab, Hornby, Ontario, Canada) and then developed with Konica immunostain HRP-1000. The PSA-positive proteins of LNCaP culture medium (LN-C7, 28.5 kDa) and benign prostate hypertrophy tissue (BP-C5, 29 kDa), prepared as described, were used for the surface plasmon resonance analysis.

Surface plasmon resonance analysis

Surface plasmon resonance was measured with an SPR670 system (Nippon Laser Electronics, Nagoya, Japan) using the sensor chip formed with self-assembly membrane by dithiodibutyric acid. After activating with ethyl-dimethylaminopropyl-carbodiimide hydrochloride and *N*-hydroxysuccinimide, the sensor chip was equilibrated with a flow buffer (10 mM phosphate buffered saline, pH 7.2) at a flow rate of 15 ml/min at 25°C. Then the anti-human PSA antibody (1:50 dilution) was loaded and covalently fixed onto the sensor chip. The remaining active residues on the chip were blocked with successive treatment of 1 M ethanolamine (pH 7.2) and 100 μg/ml of bovine serum albumin. After loading the PSA-positive fractions, 0.1 M H_3PO_4 was injected to wash the nonspecific binding materials out. To the antibody-selected PSA ligands, 500 μg/ml of two lectins, SNA and MAA (EY Laboratories), were loaded and reacted for 4 min (association phase), followed by washing with the flow buffer (dissociation phase).

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Abbreviations

AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; HPLC, high-performance liquid chromatography; LcH,

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Lens culinaris; MAA, *Maackia amurensis* agglutinin; MALDI-TOF MS, matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry; PA, pyridylamine; PSA, prostate-specific antigen; SNA, *Sambucus nigra* agglutinin.

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